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Copper deficiency increases fibulin-5 (DANCE/EVEC) but decreases cytochrome C oxidase VIb subunit expression in rat heart

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Abstract

It has been well documented that dietary copper (Cu) deficiency causes a hypertrophic cardiomyopathy in rodent models. However, a possible alteration in gene expression has not been fully examined. The present study was undertaken to determine the effect of Cu deficiency on protein profiles in rat heart tissue with the combination of the isotope-coded affinity tag (ICAT) method and Western blotting analysis. Male Sprague–Dawley rats were fed diets that were either Cu-adequate (6.0 μg Cu/g diet $n = 6$) or Cu-deficient (0.3 μg Cu/g diet $n = 6$) for 5 week. The ICAT analysis suggested that high-salt buffer (HSB) protein profiles from heart tissue of Cu-deficient rats were different from those of Cu-adequate rats; seven major protein species differed by more than a 100% increase or a 50% decrease. With three available antibodies, our Western blotting analysis confirmed that there was an 85% increase in fibulin-5 (also known DANCE/EVEC) and a 71% decrease in cytochrome C oxidase (CCO) VIb subunit, but no change in succinate dehydrogenase complex (also known complex II) IP subunit in Cu-deficient rat heart. Collectively, these data may be useful in deciphering the molecular basis for the impairments of function related to the hypertrophic-cardiomyopathy of Cu-deficient rats.
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Keywords: Copper deficiency; Heart; Fibulin-5; Cytochrome C

1. Introduction

Dietary copper (Cu) is an essential transition metal required for the activity of multiple mammalian enzymes such as Cu/Zn-superoxide dismutase, lysyl oxidase and ceruloplasmin [1,2]. The diversity of functions and tissue expression of cuproenzymes suggest multiple roles for this essential nutrient. An inadequate intake of Cu restricts the activity of cuproenzymes, and Cu deficiency has been implicated in a number of conditions including cardiomyopathy, impaired immune function and higher cancer risk [3–5]. It has been well documented that dietary Cu deficiency in the growing rat results in a number of cardiovascular disorders such as concentric cardiac hypertrophy which is a consequence of a variety of pathophysiological stimuli [6–11]. This is thought to be due in part to a reduction in the activity of the cuproenzyme lysyl oxidase, which is required in collagen and elastin crosslinking in the extracellular cardiac matrix. It has been suggested that this reduced activity could alter the loading of the myocytes and lead to hypertrophy [6–8]. However, other studies have shown that treatment of rats with the lysyl oxidase inhibitor β-amino proprionitrile reduces myocardial tissue stiffness but does not alter heart size and ventricular wall fragility [9,10]. These physiological changes likely depend primarily on the decreased activity of cuproenzymes, but several studies have demonstrated that cardiac hypertrophy

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observed in Cu-deficient male rats may involve the dysregulation of numerous genes such as reactivation of certain proto-oncogenes [11,12]. Cardiomyocytes are terminally differentiated and lose their ability to proliferate soon after birth. At the cellular level, cardiac hypertrophy is associated with an increase in cell size and protein synthesis. In view of the differential gene expression related to cardiac hypertrophy caused by Cu-deficiency, it is logical to assume that cardiac transcription factors play a key role because they directly regulate a number of cardiac genes that are involved in cardiac hypertrophy [13–15]. A high-salt buffer extraction [16] is a well accepted procedure to enrich nuclear transcription factors and other cellular proteins from other organelles such as mitochondria.

The isotope-coded affinity tag (ICAT) reagent method has been recently established for concurrent quantification and identification of expressed proteins in complex mixtures [17]. The heavy (contains d8: eight deuteriums) or light (contains d0: no deuteriums) ICAT reagents are coupled to the cysteine residues in the protein samples from two treatments, respectively, and the relative abundance of the proteins in the two samples is determined by comparing the intensity of the identical peptide peak pair as defined by the isotopic ratio of the light and heavy reagents [17]. Therefore, analysis by ICAT reagent method provides global views (rather than one at a time) of expressed protein levels related to their physiological functions. This report demonstrates, for the first time, the usefulness of combining the ICAT reagents method and Western blotting analysis in the study of protein expression affected by dietary nutrition treatment. The data provide new insights into the expression of fibulin-5 and cytochrome C oxidase VI b subunit related to the hypertrophic-cardiomyopathy of Cu-deficient rats.

2. Material and methods

2.1. Animals and diets

Experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* [18] and approved by the Animal Care Committee of the Grand Forks Human Nutrition Research Center.

The present study used the same animal samples as described in our previous report [19].

Briefly, 12 male, three-week-old, weanling Sprague–Dawley rats (Charles River/Sasco, Wilmington, MA) were divided into two dietary groups. Diets were composed of 940 g of copper (Cu)-free, iron (Fe)-free basal diet (catalog #TD 84469, Teklad Test Diets, Madison, WI); 50 g of safflower oil; and 10 g of Cu–Fe mineral mix per kg of diet. The basal diet was a casein- (200 g/kg), sucrose- (386 g/kg), cornstarch- (295 g/kg) based diet containing all known essential vitamins and minerals except for Cu and Fe [20]. The mineral mix contained cornstarch and Fe with or without Cu, and provided 0.22 g of ferric citrate (16% Fe) and either 0 or 24 mg of added $CuSO_4 \cdot 5H_2O$ per kg of diet. These formulations were intended to provide a severely Cu-deficient diet (CuD) containing only Cu present in the basal diet and a Cu-adequate diet (CuA) containing 6 mg/kg of diet. Triplicate dietary analyses (see below) of each diet indicated average Cu concentrations of 0.28 and 6.20 mg of Cu/kg of diet for the CuD and CuA diets, respectively.

Analysis of dietary Cu was performed by dry ashing of the diet sample [21], dissolution in aqua regia and measurement by atomic absorption spectroscopy (model 503, Perkin Elmer, Norwalk, CT). The assay method was validated by simultaneous assays of a wheat flour reference standard (National Institute of Standards and Technology, Gaithersburg, MD) and a dietary reference standard (HNRC-1A) that was developed by the Grand Forks Human Nutrition Research Center.

After the rats consumed their respective diets for 5 weeks, each rat was anesthetized with an intraperitoneal injection of thiobutabarbital sodium (Inactin, Research Biochemicals International, Natick, MA; 100 mg/kg body weight). Blood was withdrawn from the inferior vena cava into EDTA-treated test tubes and hemoglobin and hematocrit were determined with a cell counter (Cell-Dyn, Model 3500CS, Abbott Diagnostics, Santa Clara, CA). The median lobe of the liver was excised for mineral assays. Liver Cu and Fe concentrations were determined by lyophilizing and digesting organ samples with nitric acid and hydrogen peroxide [22] and measuring Cu concentration by inductively coupled argon plasma emission spectroscopy (Model 1140, Jarrell-Ash, Waltham, MA).

Hearts were excised and placed in phosphate buffered saline (PBS) on ice for subsequent protein extraction, described below.

2.2. Preparation of high-salt buffer (HSB) protein extract

Unless otherwise indicated, all operations were performed at 4 °C. HSB protein extracts were prepared by a generally accepted procedure [16]. Fresh tissues from heart muscle were finely minced in phosphate buffered saline (PBS) and centrifuged at 532×*g* for 5 min. The pellets were lysed in lysis buffer (20 mM *N*-[2-Hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), leupeptin (10 mg/l) and 0.1% Nonidet P-40) in a Wheaton Dounce homogenizer. Nuclei and other organelles were collected by centrifugation at 532×*g* for 5 min, suspended in lysis buffer containing 500 mM NaCl, gently rocked for 1 h, and then centrifuged at 15,000×g for 15 min. The supernatant was designated the HSB protein extract and kept at −80 °C.

2.3. Isotope-coded affinity tag (ICAT) analysis

Acetone (6 volumes of protein sample) was slowly added to a HSB protein extract pool containing equal amount of

protein from 6 different rats of a given treatment (either Cu-deficient or Cu-adequate group). The mixtures were kept at −20 °C overnight, and then centrifuged at 15,000×*g* for 30 min. The pellets were re-suspended in 6 M urea solution (6 M urea, 0.1% sodium dodecyl sulfate (SDS) and 0.25 mM phenylmethylsulfonyl fluoride (PMSF)). Protein concentrations were determined by Bio-Rad protein assay (Bio-Rad Labs, Hercules, CA) with various dilutions of bovine serum albumin (BSA) as standards. Protein samples were sent to the University of Victoria (Victoria, Canada) for comparison of the protein profiles of Cu-deficient and adequate rat hearts via ICAT analysis (service contract).

2.4. Western blotting analysis

Briefly, equal amounts (1 or 5 μg per lane) of HSB protein extract were resolved over 10% or 14% Tris[hydroxymethyl]aminomethane-glycine gels under denaturing and reducing conditions and electroblotted onto hydrophobic polyvinylidene difluoride (PVDF) membranes (Invitrogen, Carlsbad, CA). The identical SDS gels (after transferring protein to PVDF membrane) were stained with Coomassie Blue to ensure equal loading because there was always a certain percentage of protein still remaining in these gels [19]. Membrane blots were blocked in phosphate-buffered saline (PBS) – 0.05% Tween (v/v) supplemented with 1% (wt/v) nonfat dry milk (BioRad, Hercules, CA) at room temperature (RT) for 1 h. Membranes were probed with anti-CCO VIb subunit antibody, Complex II IP subunit antibody (Molecular Probes, Eugene, OR) and fibulin-5 (DANCE/EVEC) antibody (gift of Dr. Nakamura, Kyoto, Japan) at final concentrations of 1 μg/ml, 2 μg/ml and 1:200 dilution, respectively, for 1 h at RT in blocking solution. Membranes were washed (2 × 1 min, 1 × 15 min and 2 × 5 min) and then incubated with an anti-mouse (1:3000 dilution) or anti-rabbit (1:5000 dilution) horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in blocking solution for 1 h at RT. The blots were washed as above, and protein image signals were detected and quantified by using an existing chemiluminescent (ECL) plus kit (Amersham Pharmacia Biotech, Piscataway, NJ) with the Molecular Dynamics Image-Quant system (Sunnyvale, CA).

2.5. Statistical analysis

Results are given as means ± SD. Student's *t*-test for unequal variances was used to compare data between the two dietary treatments. Differences with a *P*-value < 0.05 were considered significant.

3. Results

As described in our previous report [19], the general features of Cu-deficient (CuD) vs Cu- adequate (CuA) rats (mean ± SD, $n = 6$) are body weight (g) (270.8 ± 21.7 vs 321.4 ± 29.2) ($p < 0.05$); liver Cu (μg/g) (1.7 ± 0.3 vs 13.8 ± 2.4) ($p < 0.001$); liver Fe (μg/g) (451.5 ± 104.7 vs 310.4 ± 80.2) ($p < 0.05$); hemoglobin (g/l) (74.5 ± 9.6 vs 200.8 ± 108.4) ($p < 0.05$); hematocrit (%) (22.7 ± 2.8 vs 46.5 ± 4.2) ($p < 0.001$). To determine the effect of Cu-deficiency on the gene expression linked to a hypertrophic cardiomyopathy in rat, we applied the isotope-coded affinity tag (ICAT) method and Western blotting analysis to define the change of protein profile in rat heart. Through a side-by-side sample preparation, we had both CuD and CuA high-salt buffer protein pool extracts analyzed by the ICAT method. Compared with Cu-adequate hearts, there were seven major protein species that were increased 100% or decreased 50% in Cu-deficient rat hearts (Fig. 1, Table 1). With three available antibodies, our Western blotting anal-

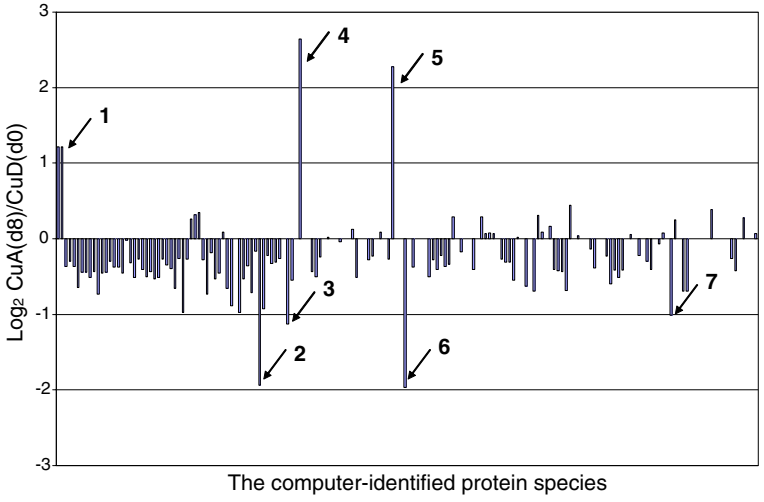

Fig. 1. The comparison of protein abundance (ratio) between CuA (d8: eight-deuterium labeled) and CuD (d0: no deuterium labeled) protein extracts analyzed by ICAT method demonstrated the difference in numerous protein species. There were seven major protein species differed by more than one fold (a 100% increase or a 50% decrease) as described in Table 1.

Table 1
The comparison of protein abundance in CuA (d8: eight-deuterium labeled) and CuD (d0: no deuterium labeled) protein extracts

GenBank Accession_ id	Gene name	$\log_2$ CuA/CuD[a]
(1) NM_025628	CCO VIb subunit	1.21472
(2) NM_011812	Fibulin-5 precursor (DANCE/EVEC)	−1.9385
(3) AK003533	Complex II IP subunit	−1.1285
(4) XM_141716	Similar to Tho2 (ribosomal protein) (Mus musculus)	2.64776
(5) NM_023913	Inositol-requiring 1alpha (yeast)	2.27195
(6) XM_136178	Similar to protein phosphatase1 (Mus musculus)	−1.96873
(7) XM_155352	Hypothetical protein XP_155352	−1.01597

[a] $\log_2$ CuA/CuD represents in the relative ratio comparison in log 2 scale.

ysis demonstrated that there was an 85% increase of Fibulin-5 (DANCE/EVEC) and a 71% decrease of cytochrome C oxidase (CCO) VIb subunit in Cu-deficient rat heart, but no change in the expression of complex II IP subunit (Fig. 2).

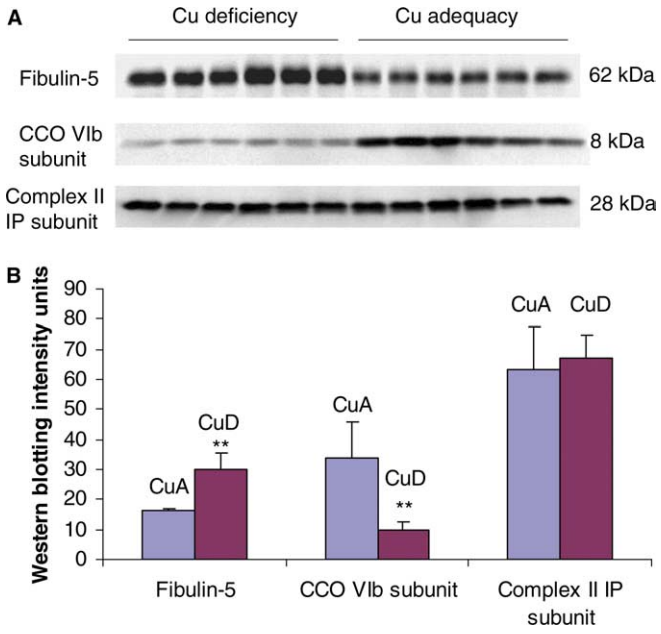

Fig. 2. Effect of copper (Cu) deficiency on protein expression of fibulin-5 (DANCE/EVEC), CCO VIb subunit and complex II IP subunit. (Panel A:) Western blotting analysis of heart high-salt buffer (HSB) protein extracts from rats fed either a Cu-deficient diet (CuD) or a Cu-adequate diet (CuA) for 5 weeks (six rats per group). For the detection of fibulin-5 and CCO VIb subunit, each lane was loaded with 5 μg of protein, and for the detection of Complex II IP subunit, each lane was loaded with 1 μg of protein. The membranes were probed with their respective antibodies individually, the size of the immunoreactive bands was estimated from regression analysis using pre-stained molecular markers (Invitrogen, Carlsbad, CA). (Panel B:) Image signals representing the protein abundance were quantified and analyzed with Molecular Dynamics ImageQuant system (Sunnyvale, CA). Values are means ± SD, $n = 6$. **Different from CuA group, $P < 0.003$.

4. Discussion

Body weight, hematocrit, hemoglobin, and liver Cu were lower, and liver Fe was higher, in CuD than in CuA rats, which are characteristic of Cu-deficient rats, and it has been documented that Cu deficiency represented by such changes causes a significant depression of Cu concentration in the heart as well [23,24]. Our previous studies suggested an additional feature of altered collagen metabolism and an increase in single-stranded cytosine-rich DNA binding in Cu-deficient rat heart [19]. Recently, large-scale expression analyses have indicated that not only fetal genes but also genes involved in signaling pathways and energy metabolism have been altered in hypertrophied hearts [25,26]. We hypothesized that the change of protein species may explain the basis of Cu-deficient hypertrophic rat heart. In the present study, we further examined the effect of Cu-deficient diet on differential gene expression in the same rat hearts described in our previous experiments [19].

With ICAT analysis, we detected seven major protein species with more than one fold difference (a 100% increase or a 50% decrease) when the HSB protein profile of Cu deficiency was compared with that of Cu adequacy. Changes in 4 of these 7 protein species could not be confirmed by Western blotting because protein identification was inconclusive. These 4 protein species are either computer-generated hypothetical proteins or predicted yeast homologues, and thus no antibodies are available for detection of the endogenous protein. We, therefore, focused on changes in the 3 protein species for which the protein identifications and functions were documented.

First, with Western blotting analysis, we demonstrated that Cu deficiency up-regulated the expression of fibulin-5, which is consistent with the data from ICAT method. Fibulin-5 (also known as DANCE or EVEC), a secreted protein of 448 amino acids, is an integrin-binding extracellular matrix protein that mediates endothelial cell adhesion; it is also a calcium-dependent elastin-binding protein that scaffolds cells to elastic fibers, thereby preventing elastinopathy in the skin, lung, and vasculature [27]. It is strongly expressed by developing arteries [28], being most pronounced in the vascular smooth muscle cells (VSMCs) of the fetal arterial vasculature, and is downregulated in most adult vascular beds [29]. However, fibulin-5 gene expression is reactivated in plaques of a mouse model of atherosclerosis and is dramatically upregulated early in the course of neointimal formation after balloon injury of the rat carotid artery [29]. Our present finding of the up regulation of fibulin-5 expression in Cu-deficient rats is generally consistent with the finding of larger atherosclerotic lesions in aortas of Cu-deficient/marginal rats [30,31], and suggests a possible mechanism by which Cu influences the vascular response to atherosclerotic injury. That this increase was found in heart suggests that fibulin-5 may play a role in compensating for the hypertrophic cardiomyopathy that is observed in Cu deficiency.

The extracellular superoxide dismutase (ecSOD) plays an important role in modulating nitric oxide bioactivity by protecting nitric oxide from the superoxide anion in the vascular extracellular space, especially in pathological states, such as atherosclerosis and hypertension where the superoxide anion is increased. More recently, it has been demonstrated that the interaction between fibulin-5 and (ecSOD) is required for ecSOD binding to vascular tissues, and regulating vascular superoxide anion levels [32]. Thereby, the up-regulation of fibulin-5 in Cu-deficient rats suggests a protective role, and represents a novel feedback pathway for controlling vascular redox state in the extracellular space in Cu-deficient hypertrophic cardiomyopathy in which oxidative stress is increased [3,8,12,33].

The other important findings in this study were the decrease of CCO VIb subunit and the lack of change of complex II IP subunit in Cu-deficient rat hearts. Cytochrome c oxidase is the terminal enzyme of the electron transport chain in mitochondria and the site where oxygen is utilized in respiration; it plays an essential role in energy supply. However, the understanding of CCO regulation is difficult because of the varying genetic origins of the subunits of this multi-subunit enzyme. A decrease in the activities of Cu enzymes such as CCO has been reported in Cu-deficient rats [33]. The decrease in CCO activity concomitant with Cu deficiency has been ascribed either to a control on the synthesis of the nuclear- or mitochondrial-encoded subunits or to the failure of assembly of the subunits during scarcity of copper [34]. Basis on our experience, the HSB protein extract contained more than 70% of total cellular mitochondrial protein because the low viscosity/density glycerol buffer was used during the centrifugation in the present study. Our present findings provided direct evidence to support the hypothesis that CCO peptides are decreased in Cu-deficient rat hearts [34–36]. However, the findings with the complex II IP subunit differed between the Western blot method, which showed no effect of copper deficiency, and the ICAT analysis, which suggested a 120% increase in expression of this protein with copper deficiency. ICAT analysis relies on the comparison of the intensity of the identical peptide peak pair as defined by the isotopic difference in the light (d0) and heavy (d8) reagents, and extrapolation of peptide to protein species based on amino acid sequence similarity. Therefore, ICAT analysis is the first step for studying the protein expression affected by Cu-deficiency, but it is necessary to use a second approach such as Western blotting analysis to confirm ICAT data. Complex II and CCO are both parts of the mitochondrial electron transport chain [37], and the difference in their protein expression demonstrates a specific effect of Cu deficiency on CCO peptide regulation.

Taken together, our present findings demonstrate for the first time an important role of fibulin-5 expression in response to Cu deficiency, and suggest a critical biological function of Cu in scaffolding cells to elastic fiber, and preventing elastinopathy in the rat heart. Furthermore, these data provide the direct evidence that the level of CCO peptide is decreased in Cu-deficient rat hearts.

5. Abbreviations

CCO	cytochrome c oxidase
CuA	Cu-adequate diet
CuD	Cu-deficient diet
DANCE	developmental arteries and neural crest EGF-like protein
ecSOD	extracellular superoxide dismutase
EVEC	embryonic vasculature and EGF like repeats contained in the predicted protein structure
HSB	high-salt buffer
ICAT	isotope-coded affinity tag
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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